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Research Article

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Analytical Biodegradation of Azo Dye (Remazol Red RB) by *Bacillus cereus*

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ABSRACT

Synthetic dyes are extensively used in textile dyeing, paper, printing, colour, photography, pharmaceutics, cosmetics and other industries. The bacterial isolate was isolated from contaminated effluent soils and processed by serial dilution and spread plate technique, identified by morphological and biochemical test followed by standard protocols, molecular characterization by16S rRNA gene sequencing and growth curve studies. Biodegradation was monitored by analyzing the degraded products by using thin layer chromatography (TLC), Fourier Transform Infra-Red (FTIR) and High Performance Liquid Chromatography.

Keywords: Remazol red RB; Bacillus cereus; TLC; FTIR; HPLC analysis

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INTRODUCTION

The textile industry is one of the greatest generators of liquid effluent pollutants, due to the high quantities of water used in the dyeing processes. Moreover, processing stages and types of synthetic dyes applied during this conversion determine the variable wastewater characteristics in terms of pH, dissolved oxygen, organic, and inorganic chemical content etc [1,2]. It is estimated that 280,000 tonnes of textile dyes are discharged in such industrial effluents every year worldwide [3]. azo dyes make up about 70% by weight [4] of all known dyestuffs in the world, making them the largest group of synthetic colorants and the most common synthetic dyes released into the environment [5-7*]*. Azo dyes accounts for the majority (3000 different varieties) of all textile dyestuffs produced because of the ease and cost effectiveness of their synthesis, their stability and the variety of colors available compared to natural dyes [8]. They are extensively used in the textile, paper, food, leather, cosmetics and pharmaceutical industries [9,10]. Many synthetic azo dyes and their metabolites are toxic, carcinogenic, mutagenic, leading to potential health hazard to humankind [11,12]. Several physicochemical methods have been used for the removal of dyes from wastewater effluent [13]. (Jadahv et al.) However, implementation of physical/chemical methods have inherent drawbacks of being economically unfeasible (more energy and chemicals), unable to completely remove the recalcitrant azo dyes and/or their organic metabolites, generating a significant amount of sludge that may cause secondary pollution problems, and involving complicated procedures [14,15]. For the biological treatment of the wastewater containing dyes, microbial or enzymatic decolorization and degradation is an eco-friendly cost-competitive alternative to chemical decomposition process and could help to reduce this enormous water consumption [16,17]. Pollution caused by dye effluent is mainly due to durability of the dyes in wastewater [18]. Decolourization of azo dyes by bacterial strains typically initiated by azoreductase involving anaerobic reduction or cleavage of azo bond [19]. These include bacteria, fungi, and algae, capable of decolorizing a wide range of dyes with high efficiency [20]. Unfortunately most azo dyes are recalcitrant to aerobic degradation by bacterial cells [21]. However, there are few known microorganism that have the ability to break the azo bond by reductive cleavage under aerobic conditions [22]. Numerous bacteria having potential of dye decolourization either in pure cultures or in consortia has been reported earlier [1,23,16].

MATERIALS AND METHODS

Sample Collection

The textile effluent and soil samples were collected from different sites of dyeing units located in Satravada, Andhra Pradesh. The effluent samples were collected from textile effluent in clean plastic bottles and soil samples were collected from in and around area of effluent contaminated soils randomly to a depth of 10-15 cm by using sterile spatula into clean and sterile polythene bags. It was refrigerated at 4°C for further analysis.

Dye and Chemicals

Reactive red RB dye was selected for the study. The commercial form of this dye used for colouring textile fibres and was provided by a dyeing unit, at Satravada, Andhra Pradesh, India. HPLC grade methanol, KBr, Ethyl acetate.

Isolation and Screening of Remazol Red RB Dye Degrading Bacteria

The bacterial isolates present in the textile dye effluent were isolated by serial dilution technique and spread plate method. The plates were incubated at 37°C for 24 hours. After incubation, the bacterial colonies with distinct characters was subcultured, purified and screened for the potential dye decolourization. The plate assay technique was performed for the detection of decolourizing activity of bacteria isolated from the textile dye effluent/soil. The bacterial isolates shows clear zone around the bacterial spot indicates dye degradation. The potential strains were selected by maximum zone of clearance in the screening plate [24]. In Quantitative screening the isolated organisms were grown in appropriate (Mineral salt medium) liquid medium.The Screening procedure in medium was continued until complete decolourization of dye.

Identification of dye Degrading Bacteria

The azo dye decolourizing bacteria was identified by using morphological, biochemical characterization [25] as described in standard protocol of Bergey's Manual of Determinative Bacteriology [25] and molecular characterization by 16Sr RNA gene sequencing.

Growth Studies

Growth curves of bacterial isolates were determined in minimal salt media with remazol red RB dye and without dye as control. For bacterial isolates 50 ml medium was taken in 100 ml flasks, autoclaved and then inoculated. Then cultures were incubated at 37^{o}C in a shaker at 100 rpm. An aliquot of culture was taken out at regular intervals of 0, 3, 6, 9, 12, 15, 18, 21and 24 hours. Absorbance was measured at 600 nm.

Extraction and Analysis of Degraded Products

After complete decolourization of 500 ml degraded samples was taken in 1000 ml Erlenmeyer flask and centrifuged at 4°C in cooling centrifuge at 10,000 rpm for 10 minutes. Then the culture supernatant was treated with equal amount of ethyl acetate dried over anhydrous sodium sulphate [26] and separated organic phase and aqueous phases by using separating funnel. Organic phase filterate was evaporated in the rotary evaporator or evaporated to dryness [27] (Pricelius et al.) and dissolved in HPLC grade methanol. The degraded products were analysed by TLC, FT-IR and HPLC.

Analytical Methods

Samples were withdrawn at regular intervals of time and centrifuged by high speed cooling centrifuge at 10,000 rpm for 10 minutes at 4°C temperature. The bioremediation experiments with cell free extract before and after degradation of the dye by *Bacillus cereus* were performed UV-Vis Spectrophotometer (Shimadzu UV-1800, Japan).

Biodegradation Assay by TLC

The residue was dissolved in a small volume of methanol and was utilised for TLC test. The developing solvent systems used were ethyl acetate: hexane 2:3(V/V) for products and ethyl acetate: methanol 7:3 (V/V) for residual dye. The spots of aromatic components were observed under UV light (365 nm) and other spots were observed by exposing the plates to iodine vapour in an iodine chamber and spotted [28].

FTIR Spectroscopy

Degradation products of the dyes were monitored by FTIR analysis. The sample and control were dried and mixed with KBr (1:20; 0.02 g of sample with KBr at a final weight of 0.4 g).The samples were ground, desorbed at 60°C and pressed to obtain IR- transparent pellets. The absorbance FT-IR spectra of the samples were recorded using an

FTIR (Brucker). The spectra were collected within a scanning range of 400 to 4000 cm^{-1}. The FTIR was first calibrated for back-ground signal scanning with a control sample of pure KBr and then the experiment sample was scanned. The FTIR spectrum of the non-degraded control was finally subtracted from the spectra of degraded dyes [28].

High- Performance Liquid Chromatography

Azo reactive RB dye was extracted with equal volume of ethyl acetate of solvent extraction method [29]. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in a rotary evaporator. The residue was dissolved in small volume of methanol. High Performance Liquid Chromatography (HPLC) was carried out on a Shimadzu LC solutions, Shimadzu Corporation , Made in Japan (column with Phenomenex C18 (2), 5 µm (250×4.60 mm) reverse phase column was used to separate individual compounds of intermediates that were detected using UV-Vis Detector. The mobile phase composed of 50% methanol and 50% nano pure water in ratio of 1:1 was used with the flow rate of 0.5 mL/min. The elutes were monitored at wavelength 254 nm using isocratic elution.

RESULT AND DISCUSSION

Isolation of Dye Degrading Bacteria

Samples was collected from different contaminated soil sites around the textile dye industries, these samples were subjected to serial dilution technique [30] and subsequently plated. A plate assay was used to detect decolourizing activity of bacteria by observing clear zones appearing around bacterial colonies on azo dye agar plate [31,32]. The isolated dye degrading bacteria were identified based on morphological, biochemical (Tables 1,2) and molecular characterization using the standard protocol. The sequence was further used for BLAST analysis from NCBI database to obtain 99% similarity with *Bacillus spp.* RR2 isolate was identified as *Bacillus cereus* with accession number KF908820.

Table 1: Morphological characteristics of *Bacillus cereus*

Characteristics	Bacillus cereus
shape	Rounded, reef edge
Edge	Lobate
Elevation	Raised
Size	Large
Texture	Soft
Appearance	Dull
Pigment	Non pigmented
Optical	Translucent

Table 2: Biochemical characterization of *Bacillus cereus*

Biochemical tests	*Bacillus cereus*
Oxidase	-
Catalase	+
Indole	-
Methyl red	+
Voges- proskauer	-
Citrate	-
Nitrate reduction test	+
Urease	-
H_2S production	-
Sugar utilization tests	
glucose	+
Lactose	+
Sucrose	+
mannitol	+
Gelatine liquefaction	+
Starch hydrolysis	-
Casein hydrolysis	+

Growth Curve Studies

The growth curve pattern was studied by growing the organisms in the presence of dye and comparing with the control (without dye). The growth pattern of both *Bacillus cereus* was significantly different from the control and the lag phase delayed up to 12 hours in the presence of both isolates when compared to control. The maximum growth was observed after 21hours in *Bacillus cereus.* The number of cells decreased as remazol red RB dye decolourization progressed over time. The cells become aged and lyse and the enzyme inside the cell goes out into the medium which interacts with the dye to reduce the dye colour. Growth rate was almost similar in both of isolates when compared with control. The growth pattern is shown in Figure 1.

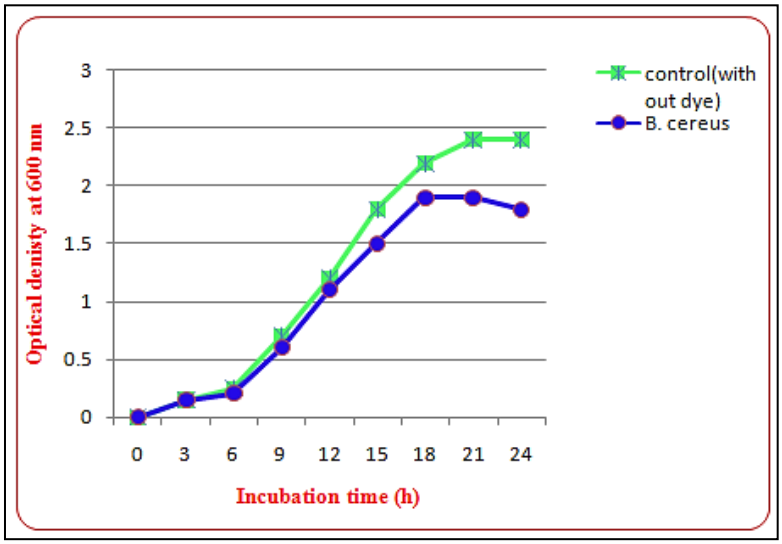

Figure 1: Growth curve of *Bacillus cereus*

Thin Layer Chromatography

The dye degradation by *Bacillus cereus s* was further supported by TLC analysis. The spot observed in the control dye solution was different from the spot observed in the culture supernatant obtained after decolourization. TLC pattern of dye decolourization by R*f* values of the dye sample 0.76, *Bacillus cereus* 0.51. This clearly indicates that decolourization was due to degradation of dyes into intermediate products. The initial step in bacterial degradation of dye is reductive cleavage of N=N (azo) bond leading to formation of colourless aromatic amines. These amines are further oxidized to simpler forms. Similarly, [33] reported that the control dye showed only one spot whereas the extracted metabolites showed two spots with *Rf* values 0.28 and 0.86 indicating the biodegradation of Metanil Yellow. TLC analysis appearance of spot and number of spots other than original dye revealed the biodegradation [34].

Fourier Transform Infrared Spectroscopy

FTIR spectrum of *Bacillus cereus* are analyzed between the scan ranges (500-3500 cm^{-1}).The FTIR spectrum obtained from the control (Figure 2) displayed a peak at 2980.01cm^{-1} indicating stretching and strong vibration of C-H bond of alkane. Peak at 1727.29 cm^{-1} and 1675.38 cm^{-1} showed C=O stretching of carbonyl group. Peaks at 1515.05 cm^{-1} showed N-O stretching vibration and strong of nitro compounds. Peaks at 1455.94cm^{-1}, 1274.84 cm^{-1} and 1123.37 cm^{-1} showed C=C stretching of medium and weak aromatic, C-N showed amine and C-O showed strong vibration of alcohol and after decolourization in *Bacillus cereus* (RR2) isolate (Figure 3) displayed a peak at 2929.13 cm^{-1} showed O-H stretching of acid. Peaks at 1660.39 cm^{-1}, 1454.77 cm^{-1}, 1384.94 cm^{-1} and 1268.28 cm^{-1}showed C=O stretching of carbonyl, C=C stretching of aromatic and stretching of C-F alkyl halide. Peaks at 1040.44 cm-1 and 954.80 cm^{-1} showed stretching of C-Namine and stretching of =C-H alkene group when compared to control. FTIR spectrum of the extracted metabolites showed absence of azo group and appearance of new groups confirming dye degradation. Similar results were reported by [35] observed that the absence of peak at 1,587 cm-1 corresponds to N=N stretching vibrations in the FTIR spectrum for degraded metabolites of Congo red. Absence of N=N indicates the cleavage of azo bond by DTS26.

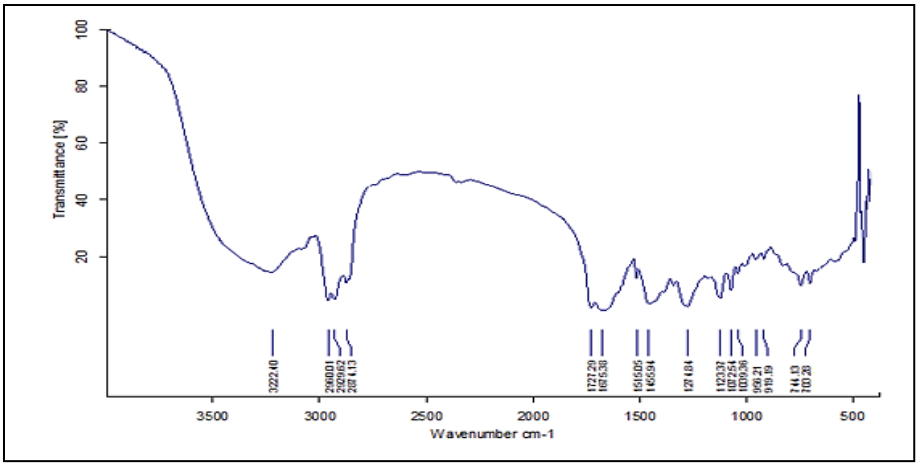

Figure 2: FTIR spectrum ofparent dyeremazol red RB (Control)

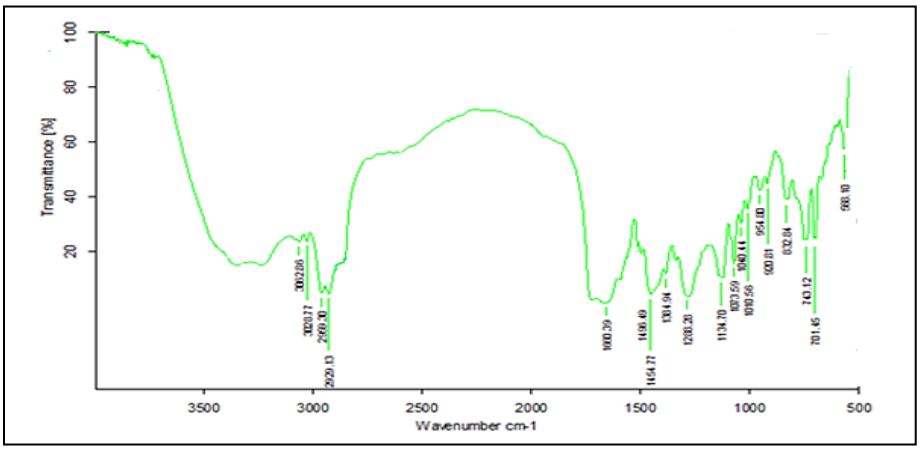

Figure 3: FTIR spectrum of remazol red RB dye degradation by *Bacillus cereus*

HPLC Analysis

The dyes were identified by comparison of the retention time in samples with standard. The HPLC elution profile of remazol red RB dye (control) showed prominent peaks at retention time 5.440, 8.812,10.949 and 12.445 minutes (Figure 4). There is a marked decrease in intensity of the peak at retention time4.838, 5.406 and 5.836 (Figure 5) in the degraded sample *Bacillus cereus* confirming the degradation of remazol red RB dye. Several peaks were also observed in the chromatogram of degraded sample indicating the production of metabolites by the isolates. Significant absence of the peaks found in the dye (control) sample and the presence of new peaks in the degraded metabolites with new retention times support the biotransformation of parent dye into molecules.

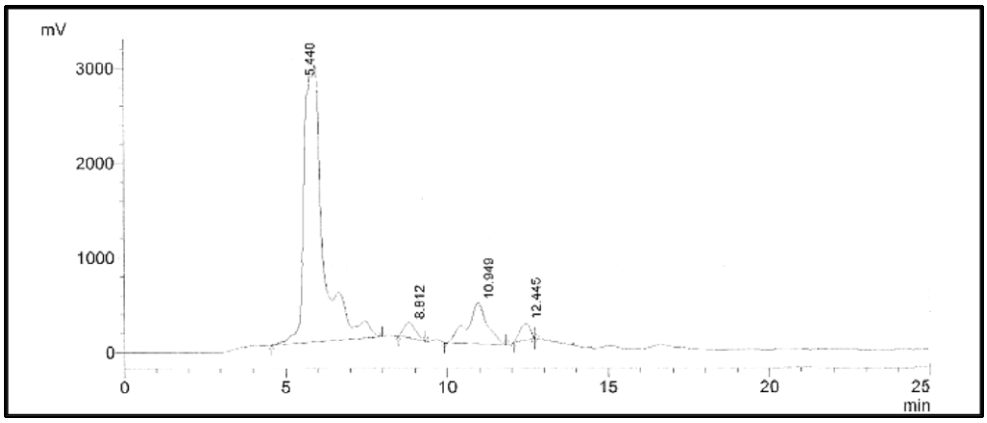

Figure 4: HPLC chromatogram of remazol red RB dye (Control)

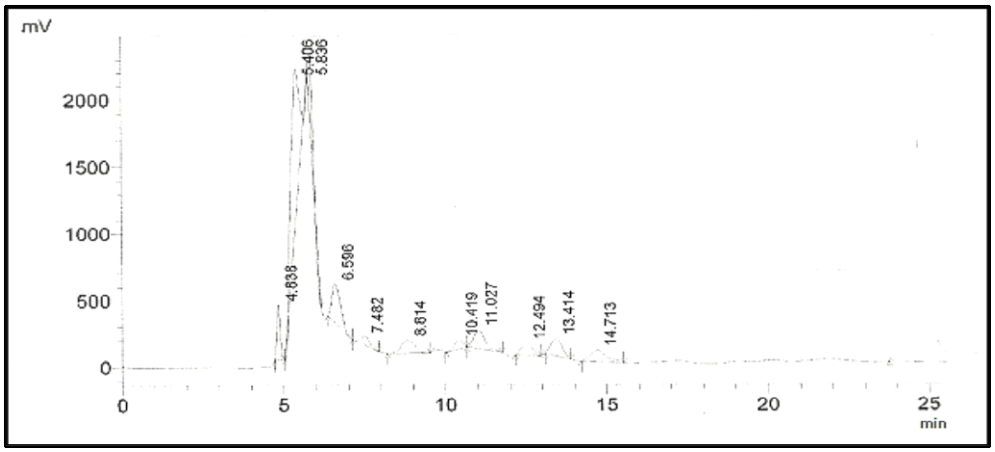

Figure 5: HPLC analysis of remazol red RB dye degradation by *Bacillus cereus*

CONCLUSION

Bacillus cereus isolated from the contaminated textile effluent sites was potential for the decolourization of remazol red RB dye. The growth curve and analytical degradation studies were revealed that the degradation of dye results into the formation of non-toxic metabolites which are ecofriendly in nature.

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